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Comparisons of genetic and morphological distance with heterosis between *Medicago sativa* subsp. *sativa* and subsp. *falcata*

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Summary

Biomass yield heterosis has been shown to exist between *Medicago sativa* subsp. *sativa* and *Medicago sativa* subsp. *falcata*. The objective of this study was to gain a better understanding of what morphological and genetic factors were most highly correlated with total biomass yield heterosis. We calculated genetic distances among nine sativa and five falcata genotypes based on amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) DNA markers. Genetic distance did not correlate with specific combining ability (SCA) or mid-parent heterosis. In contrast, a morphological distance matrix based on seventeen agronomic and forage quality traits was significantly correlated with heterosis; the agronomic traits of maturity, midseason regrowth, and autumn regrowth showed strong association with heterosis. Heterosis was also correlated with subspecies. We suggest that in many cases progeny heterosis can be accounted for by the interaction of genes controlling morphologically divergent traits between the parents. In other cases, progeny heterosis could also be due to divergence between the parents at particular genetic loci that do not control field-level phenotypic differences. Genetic distance *per se* between parental genotypes, based on neutral molecular markers, however, does not reflect the potential of individual genotypes to produce heterosis in their progeny.

Abbreviations: ADF – acid detergent fiber; ADL – acid determined lignin; AFLP – amplified fragment length polymorphisms; IVDMD – *in vitro* dry matter digestibility; MP-heterosis – mid-parent heterosis; NDF – neutral detergent fiber; RAPD – random amplified polymorphic DNA; SSR – simple sequence repeats; SCA% – specific combining ability percentage

Introduction

Medicago sativa subsp. *falcata* (hereafter referred to as 'falcata') is one of the nine proposed germplasm groups introduced into the United States; it originated in the northern latitudes of Eurasia (Barnes et al., 1977). Falcata is yellow flowered, has sickle-shaped pods, and tends to have a more decumbent growth habit and more winter hardiness than *Medicago sativa* subsp. *sativa* (hereafter referred to as 'sativa'), or purple flowered alfalfa (Lesins & Lesins, 1979). Sativa by falcata progeny show positive heterosis for total biomass yield (Westgate, 1910; Waldron, 1920; Sriwatanapongse & Wilsie, 1968; Riday & Brummer, 2002a).

The distinction between sativa and falcata germplasm is less clear using genetic markers. Kidwell et al. (1994a) found the falcata germplasm WISFAL (PI560333; Bingham, 1993) to be genetically distinct from accessions representing the other eight germplasm groups. Crochemore et al. (1996, 1998) examined morphological and random amplified polymorphic DNA (RAPD) marker data for European

and North African accessions, most of which were classified as sativa. Many of these sativa accessions, however, had large percentages of variegated flowers, indicative of falcata introgression. Although the various accessions could be placed into distinct clusters based on morphology, the RAPD markers failed to define those same relationships. Cazcarro (2000) studied forty wild alfalfa accessions from across Europe and Asia including twenty falcata and twenty sativa accessions from both allopatric and sympatric environments. The main factor associated with genetic distances based on AFLP and RAPD markers was geographical origin, rather than subspecies; however, based on morphological data the alfalfa accessions clustered into falcata and sativa groupings. These results are similar to those in oats, which showed a low correlation between morphological and genetic distance (Beer et al., 1993).

In a diversity of crop species such as chickpea, cotton, maize, pearl millet, rice, and wheat, correlations of marker heterozygosity *between* individuals (i.e., genetic distance) with heterosis have been low (Sant et al., 1999; Lui et al., 1999; Chowdari et al., 1998; Meredith & Brown, 1998; Ajmone-Marsan et al., 1998; Zhang et al., 1994; Bernardo, 1992). One reported exception is sunflower, which had high correlations between genetic distance and heterosis (r = 0.6 and 0.8) (Cheres et al., 2000). Although genetic distance often fails to correlate with heterosis, it has been successful at classifying individuals into heterotic groups (Cheres et al., 2000, Sant et al., 1999; Chowdari et al., 1998; Ajmone-Marsan et al., 1998). No such studies have been done in alfalfa, although Kidwell et al. (1994b) showed that heterozygosity of markers *within* genotypes was strongly correlated with heterosis. Kidwell et al. (1999) found no correlation between forage yield and alfalfa synthetics derived from parents that had increased molecular maker diversity.

Based on our heterosis findings in sativa-falcata hybrids (Riday & Brummer, 2002a, 2002b; Riday et al., 2002), we hypothesized that morphological divergence and/or genetic distance between parental genotypes may be correlated with progeny biomass yield heterosis.

Materials and methods

Plant materials

Fourteen genotypes (nine sativa and five falcata) were used as parents in this experiment. The nine elite sativa genotypes included ABI408, ABI311, ABI419, and ABI314 from ABI Alfalfa, Inc. (12351 W. 96 Terrace, Suite 101, Lenexa, KS 66215); C96-514, C96-673, and C96-513 from Forage Genetics (N5292 S. Gills Coulee Road, West Salem, WI 54669); and FW-92-118 and RP-93-377 from Pioneer Hi-bred International (400 Locust Street, Suite 800, PO BOX 14453, Des Moines, IA 50306). The five falcata genotypes included WISFAL-4 and WISFAL-6 from the semi-improved falcata population, WISFAL (PI560333; Bingham, 1993); C25-6, a semi-improved falcata population developed in Colorado (PI578248; Townsend, 1995); and two genotypes visually selected for vigor from plant introductions that had been planted in the field near Ames, IA: PI214218-1, derived from an accession collected in Denmark in 1954 and PI502453-1, derived from the Russian cultivar Pavlovskaya.

Crossing and field design

The fourteen selected parental genotypes were crossed in the greenhouse during autumn 1997 in a half diallel mating design, without reciprocals. Florets were hand emasculated to limit accidental self-pollination. In April 1998, seed from the 91 crosses were planted in the greenhouse. Stem cuttings of the fourteen parents were made at the same time. A total of 105 entries was included in this experiment (91 crosses and 14 parental clones). Field experiments were planted May 1998 at the Agronomy and Agricultural Engineering Research Farm west of Ames, IA and at the Northeast Research Farm south of Nashua, IA. The plot design was a quadruple α-lattice. Ten plants per plot were planted 30 cm apart within rows spaced 90 cm apart. Entries were separated by 60 cm within rows (Riday & Brummer, 2002a).

Field measurements

Total biomass yield was measured on each entry over two harvests in 1998 and over three harvests in 1999 (Riday & Brummer, 2002a). During 1999, height, growth habit, maturity, winter injury, vigor, spring regrowth, midseason regrowth, and autumn regrowth were measured (Riday & Brummer, 2002b). The following stem forage quality traits were measured

based on samples from three collections dates (October 1998, at Ames; May 1999, at Ames; and May 1999, at Nashua): *in vitro* dry matter digestibility (IVDMD), neutral detergent fiber (NDF), acid detergent fiber (ADF), acid determined lignin (ADL), crude protein, hemicellulose, cellulose, and leaf/stem ratio (Riday et al., 2002).

For each of the fourteen parental genotypes, average progeny performance was determined for each of the seventeen traits using combining ability analysis (Griffing, 1956). Using general combining ability (GCA) values, expected mean progeny performance was determined for each parental genotype (*i*) as: $\bar{X}_i = \mu + 2\mathrm{GCA}_i$ (where *i* are genotypes [1 to 14]) (Falconer & Mackay, 1996).

Heterosis calculations

Two measures of heterosis were calculated for total biomass yield of the progeny of each pair-wise combination of the fourteen parental genotypes: (i) specific combining ability percentage (SCA%) and (ii) mid-parent heterosis (MP-heterosis).

$$\mathrm{SCA\%} = \frac{\text{Progeny yield}_{ij} - (\mu + GCA_i + GCA_j)}{\mu + GCA_i + GCA_j)} \times 100 \quad (1)$$

where, *i* and *j* are parental genotypes (1 to 14), and *ij* are all ninety-one pair-wise progeny combinations, with the constraint that $i < j$.

Progeny Yield$_{ij}$ = the observed progeny yield of parental combination $i \times j$.

μ = mean yield performance of all progeny.

GCA$_i$ = General Combining Ability of parental genotype *i*.

GCA$_j$ = General Combining Ability of parental genotype *j*.

Mid-parent heterosis (%) was determined as:

$$\text{MP-heterosis} = \frac{\text{Progeny yield}_{ij} - \left(\frac{Clonal\ yield_i + Clonal\ yield_j}{2}\right)}{\left(\frac{Clonal\ yield_i + Clonal\ yield_j}{2}\right)} \times 100 \quad (2)$$

where, the terms are as defined above and

Clonal Yield$_i$ = yield based on clonal performance of parental genotype *i*.

Clonal Yield$_j$ = yield based on clonal performance of parental genotype *j*.

The difference between [1] and [2] is that SCA% is the deviation from the average performance of the progeny of specific parental genotypes, while mid-parent heterosis is based on actual parental genotype performance *per se*. For parental genotype *i*, the deviation between actual parental performance (i.e., that based on clonal measurements) and GCA (i.e., that based on progeny performance) represents 'average heterosis.' Specific combining ability (%) is a deviation from 'average heterosis', while MP-heterosis includes 'average heterosis' and SCA%.

Genetic analysis

DNA was extracted from leaf tissue of the fourteen parental genotypes using 3% CTAB buffer as described in Doyle & Doyle (1989). A 2M high salt precipitation was added to remove excess polysaccharides (Fang et al., 1990).

AFLP assays (Vos et al., 1995) used both fluorescent and radiolabed primers. The AFLP Plant Mapping Kit (Perkin Elmer) was used to generate fluorescently labeled *Eco*Rl/MSE1 DNA fragments. A fluorescent *Eco*Rl-ACA primer was used in combination with 3 MSE1 primers: MSE1-CAG, MSE1-CTC, and MSE1-CTT. Fragment analysis was performed at the Iowa State DNA Sequencing and Synthesis Facility using the ABI Prism 377 DNA Sequencer and Gene Scan Software (Perkin Elmer) as described in the AFLP Plant Mapping Kit.

Modifications to Vos et al. (1995) were used to generate radiolabeled AFLP fragments using an *Eco*Rl/*Taq* 1 restriction enzyme digest. The *Eco*Rl adapter contained a biotin label for selection of fragments containing at least one *Eco*Rl end. Following adapter ligation, the biotin label was selected using Dyna beads (Dynal). The *Taq* I selective primer AFT 24 (*Taq* I-GT) was end labeled and used in combination with three *Eco*RI primers: AFE22 (*Eco*Rl-ACC), AFE24 (*Eco*Rl-CTC), and AFE25 (*Eco*Rl-ATG). Fragments were selectively amplified using a 20ul PCR mixture containing 2μl genomic DNA digest, 0.6μl AFE primer (30 ng), 0.5μl AFT unlabeled primer (25 ng), 1μl AFT^{33}P-ATP labeled primer [5ng], 1X PCR buffer (Invitrogen), 0.5 mM dNTP's, 1.5 mM $MgCl_2$, 1U *Taq* polymerase (Invitrogen). Denatured PCR fragments were run on a 6% denaturing polyacrylamide gel. The gel was dried and an autoradiograph generated with a 3 day exposure.

Seven SSR primer pairs AFCA11, AFCA16, AFCT11, AFCT32, AFCT45, AFCTT1, and

MTLEC2A developed by Diwan et al. (1997; 2000) were amplified according to Diwan et al. (2000) with the following modifications. The 40 μL PCR contained: 1 ng DNA template; 1.2 μM unlabeled 3' and 5' primers; 150 μM dNTPs, 1.5 mM MgCl; 1 U *Taq* Polymerase (Invitrogen), 1X PCR Reaction Buffer (Invitrogen) and 0.06 μL of 3000 Ci mmol^{-1} [α-^{32}P] dATP. The annealing temperature was raised to 54 °C. The fragments were run on a 4% polyacrylamide denaturing gel cast with and run (50 w for 2 h) in a 1X extended-run TBE buffer. Each analysis was replicated three times.

Computation

All polymorphic DNA fragments were scored as present or absent across all fourteen genotypes. Similarity matrices (i.e., genetic distance matrices) were developed using the Dice coefficient (Nei & Li, 1979) calculated from (i) all polymorphic DNA fragments, (ii) AFLP fragments only, and (iii) SSR fragments only.

For each of the 142 polymorphic DNA fragments, a similarity matrix was generated to determine if heterozygosity between genotypes for a specific DNA fragment was correlated with heterosis. The similarity matrix for each polymorphic DNA fragment was calculated by inserting '0' if the fragment was heterozygous (present in one parent; absent in the other) or inserting '1' if the fragment was present in both genotypes. If the fragment was absent in both genotypes, a missing value was inserted into the matrix. For every fragment, mid-parent heterosis and SCA% values were compared with the heterozygosity score (i.e., 0, 1, or missing value) using a Mantel test (Mantel, 1967). P-values obtained from the Mantel tests were adjusted to account for multiple tests using a standard permutation test as described in Lynch & Walsh (1998).

Morphological data for each parental genotype, based on $\bar{X}_i$, was z-transformed to standardize units. A morphological dissimilarity matrix based on all traits was created from the transformed data using average taxonomic distance (Sneath & Sokal, 1973). Additionally, separate morphological dissimilarity matrices were created for each morphological trait using average taxonomic distance (Sneath & Sokal, 1973). Data transformations and matrices were calculated using NTSYS-pc software program (Rolf, 1997).

To create the SCA% and MP-heterosis dissimilarity matrices, SCA% and MP-heterosis values were z-transformed. The transformed heterosis values were then entered into a parental pair-wise comparison matrix.

To determine which similarity and/or dissimilarity matrices were highly correlated with differences between sativa and falcata genotypes, we created a subspecies similarity matrix. In the subspecies similarity matrix, within-subspecies comparisons (sativa by sativa crosses and falcata by falcata crosses) were coded '1' and between-subspecies comparisons (sativa by falcata crosses) were coded '0'. The assumption of the subspecies matrix is that all within-subspecies comparisons would show maximum similarity (1), while all between-subspecies comparisons would show maximum divergence (0).

The subspecies, SCA%, MP-heterosis, genetic distance, and morphological similarity and/or dissimilarity matrices were compared using a Mantel test. Correlation r-values and their corresponding p-values were calculated using the Mantel test feature of PopTools Microsoft Excel add-in (Hood, 2001). When a similarity matrix was correlated with a dissimilarity matrix, the sign of the correlation was switched. In cases where correlations were found with morphological distance, separate Mantel tests were performed on subspecies and SCA% similarity matrices with each of the seventeen morphological trait dissimilarity matrices. The significance of all results was assessed at the 5% probability level, after making a Bonferroni adjustment.

The neighbor-joining method (Saitou & Nei, 1987) was used to cluster the fourteen genotypes according to genetic distance (all fragments), morphological distance (all traits), SCA%, and MP-heterosis (between progeny). The neighbor-joining method was used because it tends to produce more accurate phylogenetic trees than more traditional methods such as UPGMA (Kim et al., 1992). Genotypes with greater genetic similarity, smaller morphological distances, or lower heterosis values clustered together. Dendograms and transformations were calculated and visualized using the NTSYS-pc software program (Rolf, 1997).

Results

Molecular marker analysis of the fourteen parental genotypes produced a total of 142 polymorphic DNA fragments (97 AFLP and 45 SSR). Seventy polymorphic fluorescently labeled AFLP fragments were generated using *Eco*RI-ACA in combination with

Table 1. Correlations between alfalfa (*M. sativa* L.) subspecies, yield heterosis (specific combining ability [SCA%] and mid-parent heterosis [MP-heterosis]), genetic distance (based on AFLP and SSR polymorphic DNA fragments from nine subsp. *sativa* and five subsp. *falcata* genotypes), and morphological distance (based on [i] biomass yield, [ii] stem forage quality traits, and [iii] agronomic field traits)

	Subspecies	Yield heterosis		Genetic
		SCA%	MP-heterosis	distance
Yield Heterosis				
SCA%	0.63***[1]			
MP-heterosis	0.42***	0.60***		
Genetic distance	0.32***	ns	ns	
Morphological distance[2]	0.58***	0.43**	ns	ns

***, **, * significant at the 0.001, 0.01, and 0.05 level of probability.
ns = not significant.
[1] significance levels are based on a Bonferroni adjusted Mantel-tests.
[2] biomass yield, *in vitro* dry matter digestibility [IVDMD], neutral detergent fiber [NDF], acid detergent fiber [ADF], acid detergent lignin [ADL], hemicellulose, cellulose, crude protein, and leaf/stem ratio, height, growth habit, maturity, vigor, winter injury, spring regrowth, midseason regrowth, and autumn regrowth.

MSE1-CAG (44 polymorphic fragments), MSE1-CTC (22), and MSE1-CTT (4). Radiolabeled AFLPs yielded 27 polymorphic fragments from AFT24 in combination with AFE22 (25), AFE24 (1), and AFE25 (1). A total of 45 polymorphic SSR alleles were found. Individual SSR loci yielded polymorphic alleles across all parents as follows: AFca11 (4 alleles), AFca16 (7), AFct11 (4), AFc t32 (9), AFct45 (8), AFctt1 (8), and MTLEC2A (5). The AFLP and SSR genetic distance matrices were not correlated with each other. Out of 142 distance matrices for individual polymorphic DNA fragments, none was correlated with SCA% or MP-heterosis.

The SCA% distance matrix was highly correlated with subspecies (r = 0.63, $p < 0.001$; Table 1). MP-heterosis was less correlated with subspecies (r = 0.42, $p < 0.001$) than were SCA% or morphological distance (r = 0.55, $p < 0.001$), but it was more highly correlated with subspecies than was genetic distance (r = 0.32, $p < 0.001$) (Table 1). The two heterosis measures were correlated with each other (r = 0.6, $p < 0.001$). Genetic distance based on all polymorphic DNA fragments was not correlated with either SCA% or MP-heterosis (Table 1). SCA% was correlated with morphological distance (r = 0.43 $p < 0.001$). Morphological distance was not correlated with genetic distance (Table 1).

Specific trait matrices that were highly correlated ($r > 0.6$, $p < 0.001$) with the subspecies matrix included midseason regrowth and autumn regrowth (Table 2). Weaker correlations between subspecies and

Table 2. Correlations of alfalfa (*M. sativa* L.) subspecies and yield heterosis (specific combining ability [SCA%]) with morphological dissimilarity of [i] biomass yield, [ii] stem forage quality traits, and [iii] agronomic field traits

	Subspecies	SCA%
Yield	ns[1]	ns
Forage quality traits		
IVDMD[2]	ns	ns
NDF	ns	ns
ADF	ns	ns
ADL	ns	ns
Hemicellulose	ns	ns
Cellulose	ns	ns
Crude protein	ns	ns
Leaf/stem ratio	ns	ns
Agronomic traits		
Height	0.56*	0.35*
Growth habit	ns	ns
Maturity	0.63*	0.41**
Vigor	ns	ns
Winter injury	ns	ns
Spring regrowth	ns	ns
Midseason regrowth	0.91***	0.60***
Autumn regrowth	0.71***	0.50***

***, **, * significant at the 0.001, 0.01, and 0.05 level of probability.
ns = not significant.
[1] significance levels are based on a Bonferroni adjusted Mantel-tests.
[2] biomass yield, *in vitro* dry matter digestibility [IVDMD], neutral detergent fiber [NDF], acid detergent fiber [ADF], acid detergent lignin [ADL].

specific morphological traits were found for maturity and height (Table 2). Subspecies and SCA% had a similar correlation pattern with individual morphological traits, but SCA% correlations were weaker than subspecies (Table 2).

The dendogram generated from the combined DNA fragment genetic distance matrix did not cluster parental genotypes according to subspecies (Figure 1A). The dendogram generated from the morphological distance matrix correctly separated falcata and sativa, with the exception of ABI408 and C25-6, which were accorded to the opposite subspecies group (Figure 1B). These results are consistent with previous studies, which have shown that falcata and sativa can be easily distinguished using morphological traits (Crochemore et al., 1998; Cazcarro, 2000). Both the SCA% and the MP-heterosis dendograms separated falcata and sativa into separate groups (Figures 1C and 1D).

Out of 142 distance matrices for individual polymorphic DNA fragments, none was correlated with SCA%. Three fragment matrices were correlated with mid-parent heterosis (Table 3). *Eco*RI-ACA/MSE1-CTTsize157 fragment was negatively correlated with MP-heterosis, indicating that when these polymorphic DNA fragments were heterozygous between parents, higher mid-parent heterosis was associated with their progeny. *Eco*RI-ACA/MSE1-CAGsize87 and *Eco*RI-ACA/MSE1-CAGsize67 were positively correlated, indicating that when these fragments are homozygous between parents, higher MP-heterosis was associated with their progeny (Table 3).

Discussion

Heterosis dendograms separated falcata and sativa into two groups according to subspecies (Figures 1C and 1D), evidence of a falcata-sativa heterotic pattern. The high correlation between SCA% and subspecies supports the results of the combining ability analysis for yield, which showed a general trend of positive SCA for sativa by falcata crosses (Riday & Brummer, 2002a). The higher correlation of SCA% with subspecies compared to MP-heterosis and subspecies may be a result of MP-heterosis being confounded with GCA, or 'average heterosis.' The SCA values are deviations from 'average heterosis' and therefore pick up subtle patterns in heterosis, especially if they are consistently present in crosses between a particular heterotic pattern, such as falcata and sativa in our case.

Table 3. DNA fragments scored on nine *Medicago sativa* subsp. *sativa* and five subsp. *falcata* genotypes which showed significant correlations with mid-parent heterosis for alfalfa total biomass yield (based on the progeny of a diallel cross between the fourteen genotypes)

Polymorphic DNA fragment	Sativa genotypes										Falcata genotypes					Mid-parent heterosis		SCA%	
	ABI 311	ABI 314	ABI 408	ABI 419	C96-513	C96-514	C96-673	FW-92-118	RP-93-377		WISFAL-4	WISFAL-6	C25-6	PI214218-1	PI502453-1 (r)[1]	Correlation coefficient	p-value (r)	Correlation coefficient	p-value
*Eco*RI-ACA/MSE1-CTTsize157	1	0	1	1	1	1	1	0	1		0	0	0	0	0	–0.494	<0.005[2]	–0.309	ns
*Eco*RI-ACA/MSE1-CAGsize87	0	1	1	1	1	0	0	1	0		1	1	1	0	0	0.519	<0.005	0.086	ns
*Eco*RI-ACA/MSE1-CAGsize67	1	1	1	1	1	1	0	1	1		1	1	1	1	1	0.357	0.039	–0.008	ns

1 – Fragment is present.
0 – Fragment is absent.
Correlations are based on comparisons of the heterozygosity or homozygosity of a fragment between genotype pairs and the mid-parent heterosis and SCA% values of the progeny of that genotype pair.
[1] 'r' is negative if progeny heterosis was higher when parental pairs were heterozygous for the marker; 'r' is positive when both parents have the same fragment.
[2] Significance levels are based on a permutation-test of p-values obtained from Mantel-tests of each of the 142 polymorphic DNA fragments.

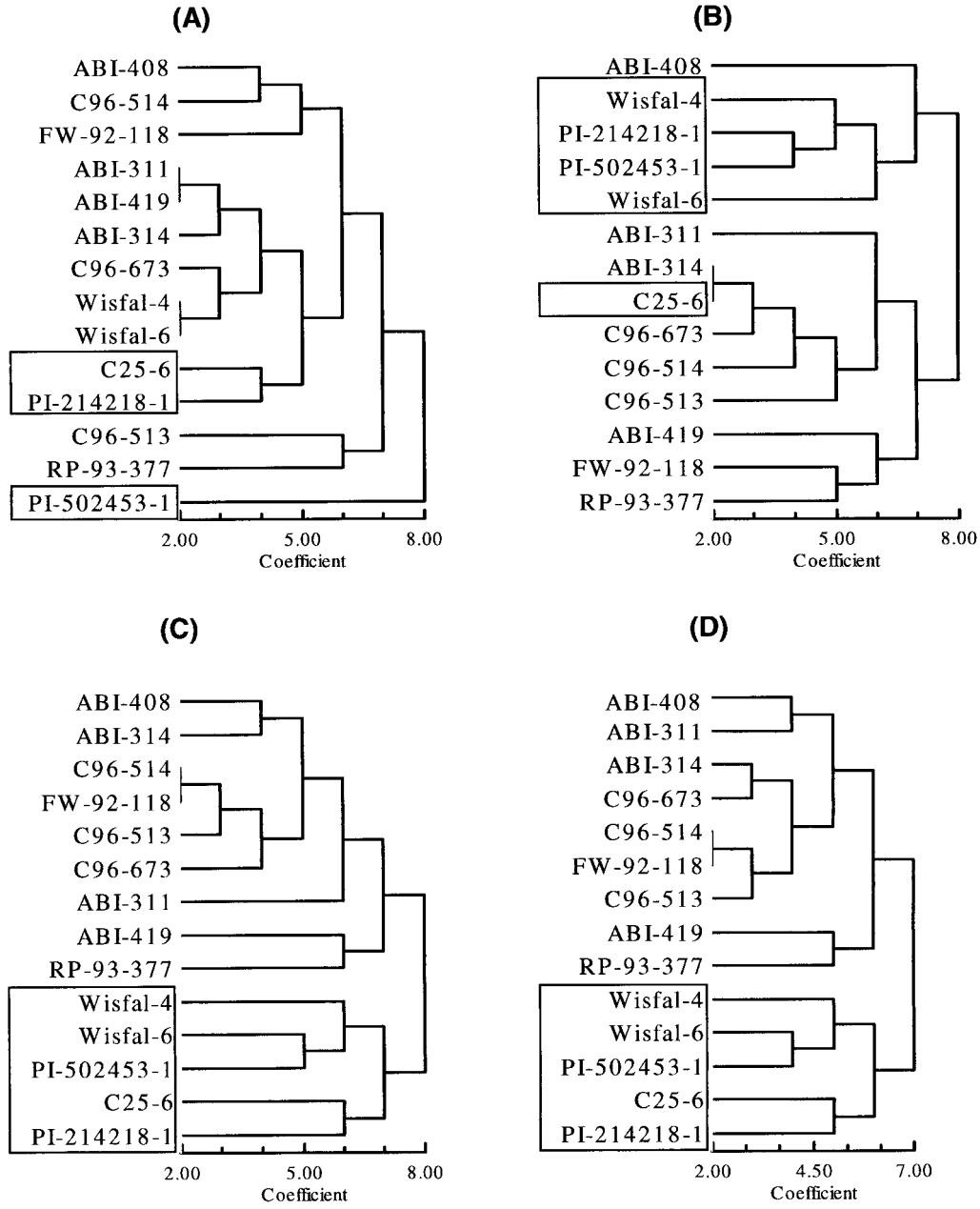

Figure 1. Neighbor-Join Dendograms of nine *Medicago sativa* subsp. *sativa* and five subsp. *falcata* genotypes based on the following distance matrices: A) genetic distance, B) morphological distance, C) SCA%, and D) mid-parent heterosis. (Sativa genotypes are: ABI408, ABI311, ABI419, C96-514, C96-673, C96-513, FW-92-118, RP-93-377. Falcata genotypes [boxed in dendogram] are: WISFAL-4, WISFAL-6, C25-6, PI214218-1, PI502453-1.)

Although we observed strong evidence for heterosis between falcata and sativa, the cause of this heterotic response is unknown. Previous studies in maize (Bernardo, 1992) and rice (Zhang et al., 1994) among others have shown that distance based on random genetic markers is a poor predictor of heterosis, possibly due to noise resulting from markers not linked directly to the trait being studied (Bernardo, 1992). In this study, markers not only did not correlate with heterosis, they also failed to place genotypes into correct subspecies groups (Table 1; Figure 1A). Although Kidwell et al. (1994a) showed that the germplasm WISFAL could be differentiated from sativa germplasm, other studies have not shown a clear distinction between diverse falcata and sativa accessions using random markers (Crochemore et al., 1996; 1998; Cazcarro, 2000). If gene flow between subspecies occurs, and the existence of *M. sativa* subsp. *varia* is evidence that it does, then clear differentiation between subspecies should not be expected. This result suggests that heterosis is a property of specific loci in the genome, which as yet have not been identified.

Heterosis expression could be related to morphological differentiation between the parental genotypes. Subspecies were more clearly clustered using morphological traits than using genetic distances based on random molecular markers (Figure 1A and 1B). Midseason and autumn regrowth, maturity, and to a lesser extent, height and growth habit appear to be the primary traits separating the subspecies (Table 2; Riday & Brummer, 2002b). These morphological traits are likely controlled by a subset of loci, which could explain why random markers, many of which are unrelated to these traits, failed to show an association between genetic distance and heterosis. One sativa genotype (ABI408) clustered morphologically with falcata genotypes, some of which produced highly heterotic progeny in crosses with ABI408 (data not shown). Thus, heterosis probably also exists due to different allelic combinations at particular loci in each parent that when brought together in hybrid combination, complement each other, resulting in heterosis expression (Bingham et al., 1994). These loci may not directly relate to observable morphological differences but could have an effect on the physiology of the plant.

One possible way to identify parents that could produce heterotic progeny would be to choose individuals, regardless of morphological or genetic similarity, from different geographic regions. Discrimination among alfalfa accessions based on geographical origin was more successful than based on subspecies status (Cazcarro, 2000). In our study, we did not have sufficient information to determine if heterosis was correlated with geographical origin of the parental genotypes. If this explanation is correct, however, we should be able to identify heterotic combinations between different sativa or falcata genotypes. Some evidence exists for intrasubspecies heterosis (Busbice & Rawlings, 1974), but more experimentation is needed to determine if this hypothesis has validity for alfalfa improvement.

Although the genetic distance matrix based on all markers did not show correlations with yield, vigor, or SCA%, weak correlations were seen for all three traits using the SSR only matrix. The reason for this could have been due to linkage of some SSR markers with QTL for biomass yield. We therefore conducted an exploratory analysis to determine if individual polymorphic fragments were associated with heterosis, realizing that the inference space was limited to these fourteen genotypes. Three AFLP showed association with MP-heterosis but none was associated with SCA% (Table 3).

Like many other crops, alfalfa had low correlations between heterotic groups and genetic distance; however, unlike many other crops, genetic distance was not correlated with morphological distance. Based on this study it appears that morphological differentiation between parental genotypes for the traits of maturity, regrowth, and autumn regrowth provides a better predictor of heterosis in alfalfa than do random molecular markers.

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